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Journal of Chromatography B, 826 (2005) 41–45
JOURNAL OF CHROMATOGRAPHY B
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Determination of atorvastatin in human serum by reversed-phase high-performance liquid chromatography with UV detection

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Received 13 May 2005; accepted 4 August 2005
Available online 16 September 2005

Abstract

A rapid and sensitive high-performance liquid chromatographic method was validated and described for determination of atorvastatin in human serum. Following liquid–liquid extraction of the drug and an internal standard (sodium diclofenac), chromatographic separation was accomplished using C18 analytical column with a mobile phase consisting of sodium phosphate buffer (0.05 M, pH 4.0) and methanol (33:67, v/v). Atorvastatin and the internal standard were detected by ultraviolet absorbance at 247 nm. The average recoveries of the drug and internal standard were 95 and 80%, respectively. The lower limits of detection and quantification were 1 and 4 ng/ml, respectively, and the calibration curves were linear over a concentration range of 4–256 ng/ml of atorvastatin in human serum. The analysis performance was studied and the method was applied in a randomized cross-over bioequivalence study of two different atorvastatin preparations in 12 healthy volunteers.

Keywords: Chromatography; HPLC; Atorvastatin; Serum; Bioequivalence study; Statins

1. Introduction

Atorvastatin (AT) [*R*-(*R**,*R**)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4[(phenylamino)carbonyl]-1*H*-pyrrole-1-heptanoic acid, is a member of the class of lipid-lowering agents called statins. The drug is potent inhibitor of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis and has been demonstrated to be effective in reducing both cholesterol and triglyceride. AT is rapidly absorbed after oral administration however, due to presystematic clearance in the gastro-intestinal mucosa and metabolism in the liver, its absolute bioavailability is approximately 12% and low plasma concentration is achieved following administration of the drug [1,2]. Thus, quantification methods of the drug in pharmacokinetic studies need to be sensitive and specific. Several analytical methods for analysis of statins [3–6] including levostatin [7,8], simvastatin [9–11], fluvastatin [12,13], pravastatin [14–21] and cerivastatin [22] in biological fluids have been published. However, there is noticeable shortage of methods described in the literature for determination of AT in the biological samples. Existing analytical methods for AT included an enzyme immunoassay [23], gas chromatography/mass spectrometry (GC/MS) [24] and high-performance liquid chromatography (HPLC) equipped with mass spectrometry detection (HPLC/MS) [25,26]. GC/MS and HPLC/MS methods are sensitive and specific with LOQ of 0.25 ng/ml, but these devices may not be available in many pharmaceutical laboratories and need highly trained persons. In addition extraction of the drug in the published HPLC/MS method [25] involved multi steps and time consuming procedures, while for large scale single dose pharmacokinetic studies a simple procedure is required. Several HPLC methods using UV detection have been reported for analysis of pravastatin [14,17,20], simvastatin [9] and lovastatin [8] in human serum, however, the present paper describes simple, fast and accurate first report of analysis of AT in human blood samples using HPLC with ultraviolet detection. This method was applied in a randomized cross-over bioequivalence study, following oral administration of 40 mg of two different atorvastatin preparations in 12 healthy volunteers.

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doi:10.1016/j.jchromb.2005.08.008

2. Experimental

2.1. Chemicals

The calcium salt of AT was from Parke–Davis (Ann Arbor, MI) and kindly provided by Arya pharmaceutical company (Tehran, Iran). The HPLC grade methanol, ethyl acetate, sodium dihydrogen phosphate, di-sodium hydrogen phosphate, sodium hydroxide and *o*-phosphoric acid were purchased from Merck (Darmstadt, Germany). Sodium diclofenac was used as internal standard (I.S.) and obtained from Sigma (St. Louis, MO, USA). All reagents used were of analytical grade except methanol which was HPLC grade. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Preparation of standard solutions

Stock solutions of AT and the I.S. were prepared by dissolving the compounds in methanol at concentrations of 400 and 40 μg/ml, respectively. The AT stock solution was diluted with methanol to working solutions ranging from 40 to 2560 ng/ml. A sodium phosphate buffer (0.1 M) was prepared in water and adjusting to pH 7.0 with 0.1 M *o*-phosphoric acid solution. All solutions were stored at 4 °C and were stable for at least 4 weeks.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of two pumps (LC-10AD), a column oven (CTO-10A), a UV–vis spectrophotometer detector (SPD-10AD) operated at wavelength of 247 nm, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. Chromatographic separation was achieved using a Shim-pack CLC-ODS analytical column (150 mm × 4.6 mm I.D.) which was packed with 5 μm particles and a Shim-pack G-ODS guard column (1 cm × 4.0 mm I.D., 5 μm particle size) with a mobile phase consisting of 0.05 M sodium phosphate buffer- methanol (33/67, v/v), adjusted to a pH of 4.0 with *o*-phosphoric acid. The column oven temperature was set at 62 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 2.5 ml/min with backpressure of 150 kg/cm^2.

2.4. Sample preparation

Blood samples were collected in disposable glass tubes (100 mm × 16 mm) and centrifuged at 4500 × *g* for 5 min. The serum samples were stored at −80 °C until analysis. To a 1 ml aliquot of the blank, calibration standard or unknown human serum sample, 100 μl volume of the I.S. and 1 ml of the phosphate buffer (0.1 M, pH 7) were added, mixed well and subjected to liquid–liquid extraction using 5 ml ethyl acetate as extracting solvent. After vortex mixing for 30 s and centrifugation (5 min at 6000 × *g*), the organic phase was removed and evaporated to dryness under stream of nitrogen at 50 °C. The residue was reconstituted in 80 μl of methanol and a volume of 20 μl was injected into the HPLC system.

2.5. Calibration curves

An amount of 100 μl from each working solutions of AT was evaporated in disposable glass tubes (100 mm × 16 mm) under a gentle stream of nitrogen at 50 °C. The residue was reconstituted in 1 ml drug-free human serum and after mixing for 30 s on a vortex mixer the samples were subjected to extraction and analysis. Calibration curves were obtained by linear least-squares regression analysis plotting of peak-area ratios (AT/I.S.) versus the AT concentrations.

2.6. Method validation

Intra-day variation was measured by assessing the different controls in replicates of six. Inter-days variation was based on repeated analysis of the same concentration controls in 10 analytical run performed on different days. The specificity of the method was investigated by the analysis of 12 human blank serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. The extraction efficacy of AT from serum was determined at concentration ranges of 4, 64 and 256 ng/ml by comparing peak areas extracted from serum with those obtained from the same amounts of unextracted solutions in methanol. The recovery of I.S. from serum was determined at a concentration of 40 μg/ml by the same method. The selectivity of the assay was evaluated by analysis of a group of potentially co-administrated drugs with AT. The limit of detection (LOD) was defined as the concentration of drug giving a signal to noise ratio of 4:1. The lower limit of quantification (LOQ) was defined as the lowest serum concentration of AT quantified with a coefficient of variation of less than 20%.

3. Results and discussion

3.1. Specificity and selectivity

Adequate chromatographic separation was obtained using the system described above. Representative chromatograms of human blank serum and human blank serum spiked with the I.S. and AT at the concentration of 4 ng/ml are shown in Fig. 1A and B, respectively. Under the chromatographic conditions described, AT and the I.S. were eluted with retention times of 3.4 and 4.1 min, respectively. No endogenous components from serum were found to interfere with the elution of the drug or I.S. Fig. 1C shows the chromatogram of serum samples obtained at 4 h after a single oral dose of 40 mg AT from a healthy volunteer. Using the same analytical conditions the selectivity of the assay was carried-out by analysis of several potentially co-administrated drugs with AT including: diltiazem, fursemide, propranalol, amiodarone, verapamil, cinnarizine, ticlopidine, glybenclamide, methyldopa, prazosin, theophylline, hydrocortisone, omeprazole, cimetidine, naproxen, indometacin, celecoxib, ibuprofen, acetaminophen, diazepam, chlordiazopoxide, oxazepam, carbamazepine, lamotrigine, aspirin and salicylic acid. All the drugs were tested

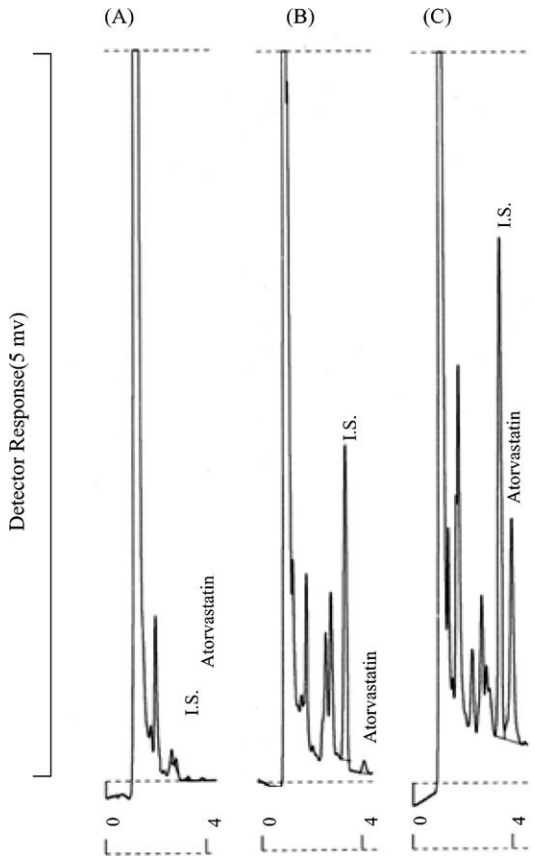

Fig. 1. Typical chromatograms obtained from an extract of: (A) human blank serum; (B) human blank serum spiked with 4 ng/ml AT and diclofenac as the I.S.; and (C) serum samples from a volunteer 4 h after a single oral dose of 40 mg drug containing 50 ng/ml of AT. The drug and I.S. were eluted at 3.4 and 4.1 min, respectively.

at concentrations range of 10–100 μg/ml. The retention times of the analyzed drugs have been shown in Table 1 and as it has been shown only ibuprofen, diazepam and celecoxib were found to be interfered with the analysis.

3.2. Sensitivity, linearity and stability

The LOD was approximately 1 ng/ml and LOQ was 4 ng/ml. The standard calibration curves were linear over the concentration ranges of 4–256 ng/ml. The correlation coefficients for calibration curves were equal to or better than 0.9965. Intra- and inter-day reproducibility were determined for calibration curves prepared on the same day ($n = 4$) and different days ($n = 10$) using pooled serum sample and the same stock solutions. Results are given in Table 2. Stock solutions of AT and I.S. were stable for at least 60 days when stored at 4 °C. The concentrations of AT in serum stored at −80 °C for 60 days and following three freeze–thaw cycles were found to be 99 ± 2% from the initial values.

Table 1
Compounds tested for interference

Substances	R_t (min)
Diltiazem	1.1
Furosemide	1.2
Propranolol	2.5
Amiodarone	5.4
Verapamil	1.2
Cinnarizine	1.5
Ticlopidine	1.1
Glybenclamide	1.3
Methyldopa	1.0
Prazosin	1.1
Theophylline	1.1
aspirin	1.6
Hydrocortisone	1.5
Omeprazole	2.3
Cimetidine	1.2
Naproxen	2.5
Indomethacin	4.7
Celecoxib	3.7
Ibuprofen	4.2
Acetaminophen	1.0
Diazepam	3.3
Chlordiazopoxide	2.0
Oxazepam	2.1
Carbamazepine	1.8
Salicylic acid	1.7
Lamotrigine	1.2

3.3. Recovery, accuracy and precision

The mean extraction efficacy of AT and I.S. from serum were found to be 95 ± 4% and 80 ± 8%, respectively. The intra- and inter-days accuracy and precision values of the assay method are presented in Table 3. The coefficient of variation values of both intra- and inter-days were all less than 11.7% whereas the accuracy of the method was 100.1–102.91% (inter-days) and 100.3–102.7% (intra-day).

Like other statins, very low blood levels are obtained after single dose administration of AT in human pharmacokinetic studies thus, for analysis of the drug using HPLC with UV detec-

Table 2
Assay linearity for determination of AT in human serum by the HPLC method

Correlation coefficient of the linear regression analysis[a] ($r \pm$ S.D.)	Slope (b) (mean ± S.D.)	Intercept (a) (mean ± S.D.)
Inter-day reproducibility ($n = 4$) 0.9965 ± 0.0025 (C.V. = 0.3%)	0.9282 ± 0.0325 (C.V. = 5.2%)	1.9409 ± 0.1812 (C.V. = 11.5%)
Intra-day reproducibility ($n = 10$) 0.9958 ± 0.0017 (C.V. = 0.34%)	0.9269 ± 0.0256 (C.V. = 7.2%)	1.9156 ± 0.2254 (C.V. = 12.6%)

r, Correlation coefficient.
[a] Linear unweighted regression, formula: $y = bx + a$.

Table 3
Precision and accuracy results of the validation

Known concentration (ng/ml)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%)
Intra-day ($n = 6$)			
4	4.11 ± 0.42	11.30	102.91
16	16.16 ± 0.57	3.56	101.0
64	64.05 ± 1.46	2.28	100.1
256	256.8 ± 3.60	1.40	100.4
Inter-day ($n = 10$)			
4	4.11 ± 0.48	11.68	102.7
16	16.26 ± 0.60	3.68	101.7
64	64.17 ± 1.50	2.35	100.3
256	256.9 ± 4.91	1.91	100.3

Accuracy has been calculated as a percentage of the nominal concentration.

tion, high efficient extraction procedure should be developed and the drug must be chromatographed without any interfering or co eluting peaks. Various extraction procedures including protein precipitation methods and liquid–liquid extraction were investigated for extraction of AT from serum. Direct protein precipitation gave low recovery with presence of endogenous impurities in the chromatograms. In spite of low solubility of AT in most of organic medium, extraction efficacy of several organic solvents including ethyl acetate, hexane, diethyl ether, dichloromethane and chloroform, either alone or in combination with different percents of 2-propanol or isoamyl alcohol were compared and the drug and the I.S. were found to be extracted from the serum into the ethyl acetate and diethyl ether. However, more efficient extract was obtained with ethyl acetate. Sample clean-up using back-extraction into acid or base, greatly decreased the interferences from serum constituents, but significantly reduced the efficacy of the extraction. Dilution of the serum samples with different amounts of various buffer solutions within different ranges of the pH and ionic strength was investigated and the results showed that addition of the same volume of phosphate buffer (pH 7.4, 0.1 M) to the serum sample improved the recoveries and significantly decreased the interferences of endogenous substances. Unlike time consuming and multi steps extraction procedure which has been used in the previously published paper [25], simple liquid–liquid extraction method was developed in our study.

The mobile phase composition was investigated and we found that the ionic strength of the buffer solution played a major role in the resolution of the chromatograms. In fact increasing the molarity of the phosphate buffer from 0.005 to 0.05 M dramatically improved the resolution of AT and the I.S. from endogenous peaks in the serum. An ultraviolet spectrophotometer scan of the drug showed maximal detection at two wavelengths of 206 and 247 nm. Wavelength of 206 nm produced increased sensitivity however; more interfering peaks emerged from the serum. Thus, the wavelength of 247 nm was selected. A number of drugs were tested to choose I.S. and diclofenac was selected considering its UV spectrum, retention time, recovery and resolution from the drug and endogenous peaks.

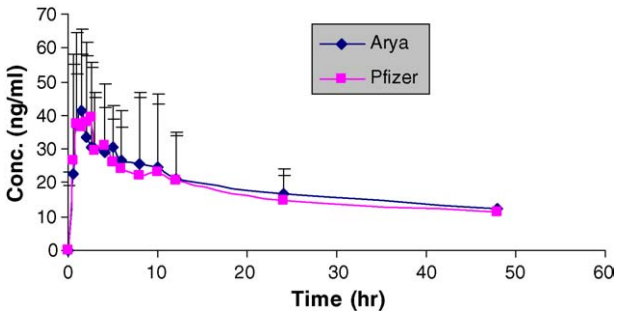

Fig. 2. Mean serum concentrations vs. time profiles of the drug for two AT preparations in 12 human volunteers after administration of a single 40 mg oral dose.

Table 4
Mean (S.D.) pharmacokinetic parameters of AT for two preparations in 12 human volunteers after administration of a single 40 mg oral dose

Parameter/prep.	Arya	Pfizer	P-value[a]
T_{max} (h)	3.2 (1.3)	2.30 (0.69)	NS
C_{max} (ng/ml)	50.1 (30.7)	47.0 (27.5)	NS
AUC_{0-48} (ng h/ml)	787.7 (548.5)	753.3 (533.2)	NS
$AUC_{0-\infty}$ (ng h/ml)	1349.2 (694.5)	1291.1 (550.3)	NS
$T_{1/2}$ (h)	35.7 (15.1)	39.9 (20.3)	NS

T_{max}, time to maximum concentration; C_{max}, maximum concentration; AUC, area under the concentration time curve; $T_{1/2}$, elimination half life.
[a] NS, no significant difference ($P < 0.05$).

3.4. Application of the method

The applicability of the described method was demonstrated in a randomized crossover bioequivalence study of two different AT preparations in 12 healthy volunteers. A single oral dose of 40 mg of AT from either Arya (Tehran, Iran) or Pfizer (Australia) pharmaceutical companies were administrated under fasting conditions and serial blood samples were collected at suitable intervals up to 48 h. Pharmacokinetic parameters including the area under the concentration time curve during the sampling period (AUC_{0-48}), the area under the concentration time curve from zero to infinity ($AUC_{0-\infty}$) and elimination half life ($T_{1/2}$) were calculated using Lagran pharmacokinetics software(Faculty of pharmacy, University of Alberta, Edmonton, Canada). The maximum serum concentration (C_{max}) and the time to reach peak (T_{max}) were directly read from the individual serum concentration–time plot. A paired t-test was used for statistical evaluation of the data and all statistical calculations were defined at the level of $P \leq 0.05$. Typical serum concentration–time profiles for two preparations are presented in Fig. 2 and obtained pharmacokinetic parameters are tabulated in Table 4.

4. Conclusion

In conclusion a simple, accurate, rapid and specific reversed-phase HPLC method using UV detection has been described for the determination of AT in serum. In this new method which has been demonstrated to be suitable for use in pharmacokinetic studies, although less sensitivity is obtained comparing to previ-

ously published LC/MS and GC/MS procedures; however, the resulted LOQ is sufficient for human pharmacokinetic studies.

Acknowledgments

This work was supported by Arya Pharmaceutical Company and in part by Kermanshah University of Medical Sciences.

References

[1] R.G. Bakker-Arkema, M.H. Davidson, R.J. Goldstein, R.J. Isaacsohn, S.L. Davignon, S.R. Weiss, L.M. Keilson, W.V. Brown, V.T. Miller, L.J. Shurzinske, J. Am. Med. Assoc. 275 (1996) 128.
[2] J.W. Nawrocki, S.R. Weiss, M.H. Davidson, D.L. Sprecher, S.L. Schwartz, P.-J. Lupien, P.H. Jones, H.E. Haber, D.M. Black, Arterioscher. Thromb. Ascular. Biol. 15 (1995) 678.
[3] X.S. Miao, C.D. Metcalfe, J. Chromatogr. A 998 (2003) 133.
[4] E.J. Park, D. Lee, Y.G. Shin, D.D. Lantvit, R.B. van Beeeman, A.R. Kinghorn, J.M. Pezzuto, J. Chromatogr. Biomed. Sci. Appl. 754 (2001) 327.
[5] T.K. Majumdar, S. Wu, S. Vedananda, F.L.S. Tse, J. Pharm. Biomed. Anal. 35 (2004) 853.
[6] S. Erturk, A. Onal, S. Muge Cetin, J. Chromatogr. B 793 (2003) 193.
[7] D. Wang-Iverson, E. Ivashkiv, M. Jemal, A.I. Cohen, Rapid Commun. Mass Spectrom. 3 (1989) 132.
[8] L.Y. Ye, P.S. Firby, M.J. Moore, Ther. Drug Monit. 22 (2000) 737.
[9] L. Tan, L.L. Yang, X. Zhang, Y.S. Yuan, S.S. Ling, Se Pu. 18 (2000) 232.
[10] H. Yang, Y. Feng, Y. Luan, J. Chromatogr. B 785 (2003) 369.
[11] H. Ochiai, N. Uchiyama, K. Imagaki, S. Hata, T. Kamei, J. Chromatogr. B 694 (1997) 211.
[12] S. Al-Rawiti, R.F. Hussein, A. Alzahrani, Ther. Drug Monit. 25 (2003) 88.
[13] H.J. Leis, W. Windischhofer, Rapid Commun. Mass Spectrom. 19 (2005) 128.
[14] K. Otter, C. Mignat, J. Chromatogr. Biomed. Sci. Appl. 708 (1998) 235.
[15] R. Seikmeier, W. Gross, W. Marz, Int. J. Pharmacol. Ther. 38 (2000) 419.
[16] C. Dumousseaux, S. Muramatsu, W. Takasaki, H. Takahagi, J. Pharm. Sci. 83 (1994) 1630.
[17] I. Iacona, M.B. Regazzi, I. Buggia, P. Villani, V. Fiorito, M. Molinaro, E. Guarnone, Ther. Drug Monit. 16 (1994) 191.
[18] Z. Zhu, L. Neirnck, J. Chromatogr. Anal. Technol. Biomed. Life Sci. 783 (2003) 133.
[19] K. Kawabata, N. Samata, Y. Urasaki, J. Chromatogr. Anal. Technol. Biomed. Life Sci. 816 (2005) 73.
[20] D.B. Whigan, E. Ivashkiv, A. Cohen, J. Pharm. Biomed. Anal. 7 (1989) 907.
[21] Z. Zhu, Neirinck L, J. Chromatogr. B 783 (2003) 133.
[22] N.V.S. Ramakrishna, M. Koteshwara, K.N. Vishwottam, S. Puran, S. Manoj, M. Santosh, Pharm. Biomed. Anal. 36 (2004) 505.
[23] Y.Y. Shum, H. Huang, G.A. Walter, A.E. Black, C.S. Sekerke, R. Homan, R.S. Newton, G.S. Nordblom, D.S. Wright, T. Chang, L.R. Whitfield, Pharm. Res. 10 (Suppl.) (1993) S415 (abstract PPDM 8467).
[24] R. Hayes, D. Minser, J.A. Jersey, B.E. Lilley, Pharm. Res. 13 (Suppl.) (1996) S47 (Abstract APQ 1180).
[25] W.W. Bullen, R.A. Miller, R.N. Hayes, J. Am. Soc. Mass Spectrom. 10 (1999) 55.
[26] M. Jemal, Z. Ouyang, B.C. Chen, D. Teitz, Rapid Commun. Mass Spectrom. 13 (11) (1999) 1003.